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A Generic Intron Increases Gene Expression in Transgenic Mice

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To investigate the role of splicing in the regulation of gene expression, we have generated transgenic mice carrying the human histone H4 promoter linked to the bacterial gene for chloramphenicol acetyltransferase (CAT), with or without a heterologous intron in the transcription unit. We found that CAT activity is 5- to 300-fold higher when the transgene incorporates a hybrid intron than with an analogous transgene precisely deleted for the intervening sequences. This hybrid intron, consisting of an adenovirus splice donor and an immunoglobulin G splice acceptor, stimulated expression in a broad range of tissues in the animal. Although the presence of the hybrid intron increased the frequency of transgenics with significant CAT activity, it did not affect the integration site-dependent variation commonly seen in transgene expression. To determine whether the enhancement is a general outcome of splicing or is dependent on the particular intron, we also produced equivalent transgenics carrying the widely used simian virus 40 small-t intron. We found that the hybrid intron is significantly more effective in elevating transgene expression. Our results suggest that inclusion of the generic intron in cDNA constructs may be valuable in achieving high levels of expression in transgenic mice.

It has been well documented that splicing can influence the production of stable mRNA in eukaryotic cells (4, 19). Alternative splicing of introns can occur in a tissue-specific (10), development stage-specific (2), or sex-specific (17) manner. *cis*-acting regulatory elements have also been found within introns (1). Introns may also separate distinct functional domains in some genes, suggesting that they may accelerate the generation of proteins with novel functions during evolution (5). These rather diverse processes utilize intervening sequences passively, as structural elements. A more direct, active role of splicing in the regulation of gene expression has also been proposed (3, 14).

Soon after the development of mammalian expression vectors, it was shown that simian virus 40 (SV40) 16S mRNA formation required the presence of at least one intron (7), suggesting that splicing might be a general requirement for eukaryotic gene expression (8). Subsequently, however, a few endogenous genes, such as hsp70 (23), were shown to lack intervening sequences, and vectors without introns were constructed that gave sufficient expression of some cDNAs (20). One commonly used vector, pSV2 (22), provides the SV40 small-t intron as part of the 3′ untranslated region. Thus, satisfactory expression is routinely obtained in tissue culture. On the other hand, poor or no expression of cDNAs is more often the rule than the exception in transgenic mice.

The ability to introduce foreign DNA into the germ line of mice has permitted molecular approaches to the understanding of biological processes at the organism level (15). A number of experiments have demonstrated that chimeric constructs often yield poor expression in the desired tissue(s) or give unanticipated expression in other tissues, or both. Genomic coding sequences, complete with introns, are more likely to be expressed in transgenics than cDNAs, due at least in part to the presence of specific regulatory elements that often reside in introns. However, limitations of size and availability often dictate that cDNAs must be used, and it is unclear to what extent the inclusion of a heterologous intron can enhance cDNA expression in transgenic mice. In this study, we have used a ubiquitously expressed promoter fused to a reporter gene to further investigate the regulatory role of splicing in transgenic mice.

MATERIALS AND METHODS

Plasmid constructions. pF3PN2 carries a 6.5-kbp *Xba*I-*Pst*I fragment containing the human histone H4 promoter and enhancer (11) inserted into the *Xba*I and *Pst*I sites of pUC18N2, a pUC18 derivative with *Not*I sites in place of the *Sma*I and *Hin*dIII sites. pF3MN2 was derived from pF3PN2 by insertion of an *Mlu*I linker (Stratagene) at the *Pst*I site. A 1.6-kbp *Hin*dIII-*Bam*HI fragment containing chloramphenicol acetyltransferase (CAT) coding and SV40 small-t intron and early polyadenylation sequences was isolated from pSV2-cat (22), *Pst*I linkered, and inserted into the *Pst*I site of pF3PN2 to generate pF3CAT. A 1.4-kbp *Xho*I-*Nae*I fragment from pMLSIS.CAT (14) containing a hybrid intron in the 5′ untranslated leader, CAT coding sequences, and the SV40 late-orientation polyadenylation site was blunt-ended with Klenow, *Mlu*I linkered, and inserted into the *Mlu*I site of pF3MN2 to generate pF3SiCAT. pF3SCAT was similarly constructed by using the 1.2-kbp *Xho*I-*Nae*I fragment from pMLSS.CAT (14).

Generation of transgenic mice. Plasmids pF3CAT, pF3SCAT, and pF3SiCAT were digested with *Not*I, and the DNAs for microinjection were prepared by standard procedures (12). FVB/N mice were superovulated by standard procedures (12), and fertilized eggs were injected with 2 to 8 fg of DNA. The microinjected embryos were then transferred into the oviducts of pseudopregnant FVB/N mice.

Preparation and analysis of mouse genomic DNA. DNA was prepared from the tailtips of 3- to 4-week old mice and analyzed by Southern blotting or by slot blot. A 2.7-kbp *Eco*RI fragment from the human histone H4 locus or a 0.7-kbp *Hin*dIII fragment containing CAT coding sequences

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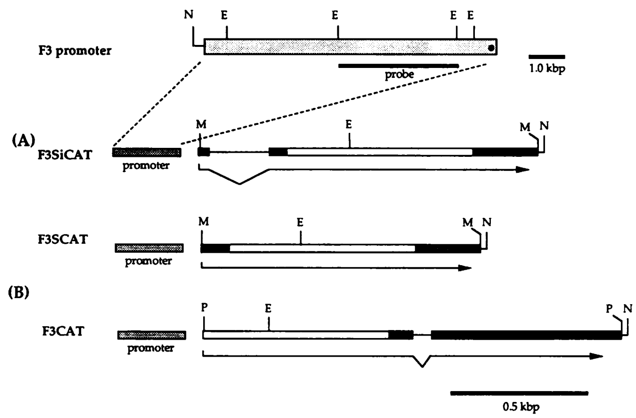

FIG. 1. Diagram of the H4-CAT constructs injected into fertilized mouse eggs. The 6.5-kbp F3 promoter region is depicted in detail at the top. The TATA box is represented as a dot at the extreme right of the promoter. (A) F3SiCAT and F3SCAT transgene fragments. (B) F3CAT transgene fragment. The F3 promoter is drawn to a different scale than the downstream regions for all three constructs. The scale bar at the upper right applies to the enlarged promoter at the top, while the scale bar at the lower right applies to the three transcription units in panels A and B. The probe was derived from CAT coding sequences or the F3 promoter fragment. Stippled box, F3 promoter; black box, intron-containing fragment; line, intervening sequence; open box, CAT coding region; grey box, polyadenylation signal-containing fragment. Restriction enzyme sites: E, *Eco*RI; M *Mlu*I; N, *Not*I; P, *Pst*I.

was used as the probe. The membranes were exposed to film or analyzed on a Betascope 603 (Betagen).

CAT assays. Tissues of 4- to 6-week-old founder animals or F_1 animals were rapidly isolated and quick frozen in liquid nitrogen. Individual tissue samples were disrupted in 1 ml of 0.25 M Tris (pH 7.5) with a Brinkmann polytron. The crude extracts were heat treated at 60°C for 15 min to inactivate endogenous acetylases. Extracts containing 100 μg of total protein (Bradford assay; Bio-Rad) were incubated at 37°C for 4 h, and the acetylated products were isolated and assayed by thin-layer chromatography by standard methods (20). Quantitation was performed on a Betascope 603.

RESULTS

Production of histone promoter-CAT transgenic mice. To evaluate the effect of splicing on gene expression in vivo, we introduced three promoter-reporter chimeric genes into transgenic mice (Fig. 1). Because we wished to make a comprehensive tissue survey of the regulatory role of splicing, all three constructions feature a human histone H4 promoter fused to the gene for CAT. This promoter has been used in our laboratory to express a number of cDNAs in a broad spectrum of mouse tissues. F3SiCAT contains a hybrid intron in the 5′ untranslated region. This 230-bp intron consists of a splice donor and some intervening sequence from the adenovirus major late tripartite leader and an immunoglobulin G (IgG) acceptor (14). F3SCAT is identical to F3SiCAT except that the intervening sequence has been precisely deleted, so that the F3SiCAT transcript after splicing would be identical to the F3SCAT primary transcript. Both F3SiCAT and F3SCAT are terminated by the SV40 late-orientation polyadenylation signal. We chose to use this hybrid intron for our expression studies in transgenics because splicing of this intron has been demonstrated to increase the expression of tissue plasminogen activator and factor VIII cDNAs in a variety of cell lines (data not shown). To compare the hybrid intron to a frequently used splicing cassette, a similar construct, F3CAT, containing the SV40 small-t intron and early polyadenylation signal downstream of the CAT coding sequence, was also introduced into transgenic mice. Because essentially all constructs bearing the small-t intron place the intron 3′ of coding sequences, we chose to retain this configuration to better contrast the hybrid and small-t introns as they are currently utilized.

Approximately 200 fertilized FVB/N mouse eggs successfully injected with the *Not*I fragments shown in Fig. 1 were implanted into pseudopregnant foster mothers. The resultant founder class offspring (F_0) were screened by Southern blot analysis of tail genomic DNA with a 2.7-kb *Eco*RI fragment from the promoter region as a probe. Transgenic animals thus identified were numbered sequentially (e.g., F3SiCAT-1 through F3SiCAT-12). The frequencies of transgenic mice obtained were 41% (12 of 29 F_0 animals) for F3SiCAT, 24% (9 of 38) for F3SCAT, and 16% (8 of 49) for F3CAT. Transgene copy numbers, determined by quantitation of slot blot hybridization intensity on a Betascope 603, varied from five to several hundred integrated copies.

Hybrid intron greatly enhances CAT expression. Homogenates were prepared from the tissues of seven independent 4-week-old F3SiCAT transgenics and nine independent 4-week-old F3SCAT transgenics. The tissues surveyed were liver, spleen, kidney, brain, thymus, testes or uterus, heart, lung, pancreas, and skeletal muscle.

F3SCAT-1 through F3SCAT-5 showed essentially no expression in all tissues tested. Trace activity (two- to fourfold over background) was detected in one or two tissues of these mice, most frequently the brain or thymus. Significant (10- to 100-fold over background) transgene expression was found in the brain of F3SCAT-6, in the testes of F3SCAT-7 and F3SCAT-8, and in the skeletal muscle of F3SCAT-9. The other tissues of these four mice had trace levels of CAT activity (Fig. 2). In sharp contrast, six of the seven F3SiCAT mice screened had significant CAT activity in almost every tissue tested (Fig. 2). The tissue distribution of transgene expression levels was different in each of these animals, presumably reflecting the influence of the particular integration site (position effect). In addition, there was no correlation between transgene copy number and the expression levels in these mice. Taken as a group, however, CAT activity in these F3SiCAT mice was most prominent in the brain, thymus, testes, heart, and lung and lowest in the liver. This pattern of expression is similar to that seen in the F3SCAT mice, though at notably higher levels, and indicates that splicing enhances transgene expression in all tissues.

F3SiCAT-3 had basal levels of CAT activity in all tissues, with trace activity in the thymus, much like the majority of F3SCAT mice. Because about 10 copies of the transgene had integrated in F3SiCAT-3, it is unlikely that the lack of expression is due to an inhibition of all transgenes. It is more likely that the transgenes had integrated into a transcriptionally silent chromosomal locus and were inactivated.

Splicing does not influence position effects. Since the frequency of animals with significant expression was much greater for F3SiCAT than F3SCAT, we asked whether splicing might reduce the position effect on expression in these animals. We examined the variance in the levels of expression in tissue samples with detectable signal over background in F3SiCAT and F3SCAT mice (Table 1). The only tissues for which a sufficient number of samples with detectable levels of expression were available were the brain

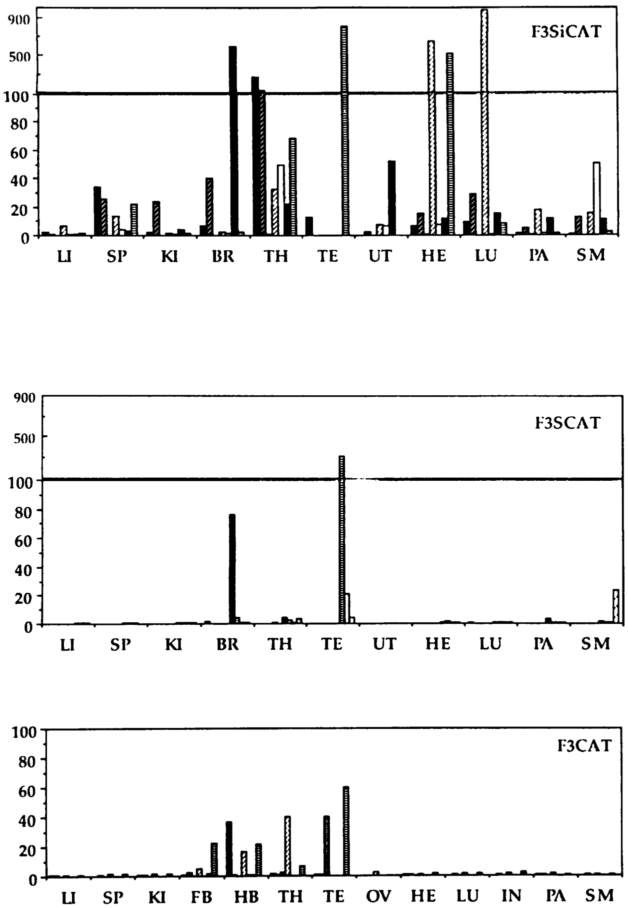

FIG. 2. CAT activity in tissues of F3SiCAT, F3SCAT, and F3CAT mice. Tissues from seven F3SiCAT lines, nine F3SCAT lines, and seven F3CAT lines were analyzed. Tissues analyzed were liver (LI), spleen (SP), kidney (KI), brain (BR), thymus (TH), testes (TE), uterus (UT), ovary (OV), heart (HE), lung (LU), pancreas (PA), and skeletal muscle (SM). The number of samples of each tissue is equal to the number of animals analyzed except for F3SiCAT testes ($n = 2$) and uterus ($n = 5$), F3SCAT testes ($n = 3$) and uterus ($n = 6$), and F3CAT testes ($n = 5$) and ovaries ($n = 2$). CAT assays were performed under standard conditions and quantitated on a Betascope 603. Activity is given in arbitrary units, equivalent to percent conversion under standard assay conditions. Extract dilutions (brought up to 100 μg of total protein with bovine serum albumin) were used where necessary to maintain the linearity of the assay, and activity values were extrapolated to standard conditions. In eight tissue samples, the activity was over 100, and these are indicated on the figure as 100. The cases are listed below, with the true activity value in parentheses: F3SiCAT-1 thymus (266), F3SiCAT-2 thymus (124), F3SiCAT-4 heart (646), F3SiCAT-4 lung (971), F3SiCAT-6 brain (593), F3SiCAT-9 testes (803), F3SiCAT-9 heart (523), and F3SCAT-7 testes (319).

and thymus. As a measure of relative variance, the standard deviation of two data sets can be directly compared when the mean value of each of the data sets is equal. When the mean values differ but are proportional to the standard deviations, as in this case, the variance is assessed independently of the mean by determining the standard deviation of the log of the values that compose each data set (16).

In thymus samples from F3SiCAT mice, the mean log of CAT activity was 3.5, with a standard deviation, or variance, of 0.80 (Table 1). In thymus samples from F3SCAT mice, the

TABLE 1. Variable expression of F3SiCAT and F3SCAT in brain and thymus[a]

Tissue and construct	CAT activity, arbitrary units (log), in strain: 1	2	3	4	5	6	7	8	9	Mean activity	SD[b] All	− High	− Low	− Median
Thymus														
F3SiCAT	26,600 (4.4)	12,400 (4.1)	85 (1.9)	3,200 (3.5)	4,920 (3.7)	2,240 (3.4)	ND[c]	ND	6,820 (3.8)	3.5	0.80	0.76	0.39	0.87
F3SCAT	ND	18 (1.3)	4 (0.6)	39 (1.6)	16 (1.2)	435 (2.6)	255 (2.4)	95 (2.0)	295 (2.5)	1.8	0.72	0.68	0.59	0.78
Brain														
F3SiCAT	695 (2.8)	4,040 (2.6)	18 (1.3)	215 (2.3)	165 (2.2)	59,300 (4.8)	ND	ND	235 (2.4)	2.6	1.1	0.54	0.96	1.2
F3SCAT	125 (2.1)	NA[d]	1 (0.0)	NA	NA	7,590 (2.9)	425 (2.6)	42 (1.6)	52 (1.7)	1.8	1.1	1.0	0.56	1.1

[a] Arbitrary units are equivalent to percent acetylation × 100 under standard assay conditions as given in Materials and Methods. When conversion was over 50%, assays were repeated with extract dilutions until reactions were in the linear range and then extrapolated to standard conditions. Background ranged from 10 to 20, and CAT activity values reflect a correction of 15 for background. Variance ratios calculated by using background values of 10, 15, and 20 differed by less than 8%.

[b] SDs were calculated for all datum points and without the high, low, and median datum points (see text).

[c] ND, not determined.

[d] NA, no activity detected on film or on Betascope.

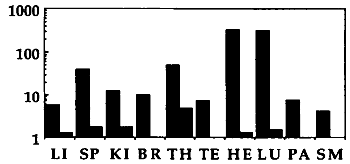

FIG. 3. CAT activity enhancement factors for tissues of F3SiCAT and F3CAT mice relative to F3SCAT mice. The left and right bars represent F3SiCAT and F3CAT, respectively. Enhancement factors were calculated by dividing the average CAT activity for each tissue in the F3SiCAT and F3CAT lines by the average CAT activity for that tissue in the F3SCAT lines. For four F3CAT tissues, the enhancement factor was less than 1; these are not depicted on the figure and are listed below with the enhancement factor in parentheses: brain (0.84), testes (0.06), pancreas (0.88), and skeletal muscle (0.14). See Fig. 2 legend for abbreviations.

mean log activity was 1.8, with a similar variance of 0.72. Thus, the effect of splicing is reflected in the increased mean value (3.5 − 1.8 = 1.7 log units, or 50-fold greater activity) and not reflected in the variance (0.80 − 0.72 = 0.08, or 1.2-fold). Similarly, standard deviations of 1.1 and 1.1 were found for F3SiCAT brain and F3SCAT brain, respectively. Our results suggest that splicing does not render transgene expression independent of position effect but rather increases the level of expression. To test the validity of the calculated values given the relatively small sample size, we determined the standard deviations for the sample sets omitting the high, low, or median datum points. The difference in variance between the spliced and unspliced data sets was not greater than 0.46 logs, or 2.9-fold, as the sample size was reduced (Table 1). Therefore, we conclude that splicing does not influence the variability of transgene expression due to position effect.

SV40 small-t intron confers a slight enhancement of transgene expression. Of the eight founder animals transgenic for the F3CAT construct containing the small-t intron, seven passed the transgene into the F_1 generation. Tissues were isolated from an F_1 animal from each of the seven lines, crude extracts were prepared, and CAT assays were performed exactly as was done for the F3SiCAT and F3SCAT samples. Five of the F3CAT lines were indistinguishable from the F3SCAT mice, showing trace or sporadic CAT activity in the brain, thymus, or testes. F3CAT-5 and F3CAT-8 evidenced a broader spectrum of transgene expression, but at levels manyfold lower than were routinely found in the F3SiCAT mice (Fig. 2). On average, inclusion of the small-t intron increased CAT expression fivefold in the brain and less than twofold in all other tissues. The hybrid intron, by comparison, enhanced gene expression in every tissue 5- to 250-fold more than the small-t intron. (Fig. 3).

DISCUSSION

It has been shown that splicing can greatly increase the levels of cytoplasmic mRNA (14). In the experiments described in this report, we have shown that the addition of a hybrid intron greatly stimulates gene expression in transgenic mice. This effect, which occurred in every tissue examined, ranged from a 5-fold enhancement in the liver and skeletal muscle to over a 300-fold enhancement in the heart and lung. A comparison of the hybrid intron and the SV40 small-t intron demonstrated that this enhancement is much more pronounced for the hybrid intron than for the SV40 small-t intron. Furthermore, we found that splicing does not abrogate integration site-dependent variation.

Although the mechanism by which splicing increases CAT activity in the F3SiCAT animals is not known, an examination of the effect in cell culture suggests an explanation. It was previously shown, using the transcription units in F3SiCAT and F3SCAT under the control of the SV40 promoter, that splicing of the hybrid intron led to a 6- to 50-fold increase in CAT activity in a number of cell lines (14). This difference in activity was reflected in the levels of cytoplasmic mRNA, and analysis of the nuclear RNAs indicated that a greater fraction of transcripts from the spliced construct than from the unspliced construct were polyadenylated. No difference in transcription rates or in cytoplasmic or nuclear RNA stability was found between the two transcripts, suggesting that the mechanism of enhancement was solely at the level of increased polyadenylation and transport to the cytoplasm. It is reasonable to predict that a similar mechanism is responsible for the enhancement seen with the hybrid intron in transgenic mice. However, due to the great abundance of RNases liberated during the isolation of nuclei from a number of tissues, as well as the significantly lower expression obtained in transgenic mice compared with that in transient transfections in cell culture, a similar assessment of polyadenylation in tissues of F3SiCAT and F3SCAT mice was not possible.

These cell culture experiments with the hybrid intron, as well as those of Buchman and Berg (4) and Hamer and Leder (8), all suggest a posttranscriptional mechanism for the splice-dependent enhancement of expression. On the other hand, Brinster et al. have reported that the complete rat growth hormone gene with its native introns is transcribed more efficiently than the cDNA form in transgenic mice (3). The presence of enhancers within the growth hormone gene introns could not be ruled out, however. There are two lines of evidence from cell culture experiments which suggest that the hybrid intron used in our experiments does not harbor an enhancer. First, nuclear run-on assays showed no difference in transcription initiation rates between the transcription units in F3SiCAT and F3SCAT driven by the SV40 promoter (14). Second, there was no enhancement of CAT activity when the hybrid intron was placed upstream of the SV40 promoter (data not shown).

Our studies comparing the SV40 small-t intron and the hybrid intron show a dramatic difference in their effect on transgene expression. Whereas the addition of the hybrid intron led to a substantial increase in gene expression in all tissues, the presence of the small-t intron had minimal effect. Recently, Palmiter et al. (18) found that placing the small-t intron 5′ of the rat growth hormone cDNA resulted in 20-fold greater expression in the fetal liver of transgenic mice than if the intron were placed 3′ of coding sequences. In agreement with what we have observed in vivo, Evans and Scarpulla (6) showed that the small-t intron placed downstream of CAT sequences elevated CAT activity only 2-fold in COS-1 and NIH 3T3 cells, whereas a rat *cyc* intron in the 5′ untranslated region resulted in a 280-fold stimulation. Interestingly, when both introns were present, CAT activity was intermediate between those with either intron alone. A possible mechanism for this inhibitory effect of the small-t intron is that its small size promotes unproductive splicing events. In cell culture experiments, the presence of the small-t intron downstream of CAT revealed cryptic splice donor sites within the CAT coding sequence, and increasing the size of the small-t intron eliminated these aberrant splices in favor of the correct splicing event (13). Taken together, these studies support our finding that transgene constructs which include the small-t intron downstream of coding sequences are not optimized for expression.

As a practical consideration, our experiments indicate that regardless of the tissue(s) that has been targeted, the inclusion of the hybrid intron in transgene constructs is an appropriate strategy to maximize expression. Since the hybrid intron stimulated the expression of tissue plasminogen activator and factor VIII in tissue culture, the enhancement seen in mice is unlikely to be specific to CAT and instead may be generally applicable to the expression of cDNAs in transgenic mice.

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